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Research article

Wound and methyl jasmonate induced pigeon pea defensive proteinase inhibitor has potency to inhibit insect digestive proteinases

Purushottam R. Lomate, Vandana K. Hivrale*

Department of Biochemistry, Dr. Babasaheb Ambedkar Marathwada University, Aurangabad 431004, Maharashtra State, India

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ABSTRACT

Wounding of plants by chewing insects or other damage induces the synthesis of defensive proteinase inhibitors (PI) in both wounded and distal unwounded leaves. In the present paper we report the characterization of inducible defensive PI from pigeon pea (*Cajanus cajan*) and its *in vitro* interaction with *Helicoverpa armigera* gut proteinases (HGP). We found that PI activity was induced in local as well as systemic leaves of pigeon pea by the wounding and methyl jasmonate (MeJA) application. Consistent induction of PI was observed in two wild cultivars of pigeon pea at various growth stages. The estimated molecular weight of inducible PI was ~16.5 kDa. Electrophoretic analysis and enzyme assays revealed that the induced PI significantly inhibited total gut proteinase as well as trypsin-like activity from the midgut of *H. armigera*. The induced PI was found to be inhibitor of trypsin as well as chymotrypsin. Study could be important to know the further roles of defensive PIs.

1. Introduction

Wounding of leaves by herbivore attack activates the expression of PI genes which leads to the production of protease inhibitor proteins [1]. Protease inhibitors are important elements of the plant defense response to insect predation [2]. Production of these inhibitors is highly regulated by a signal transduction pathway that is initiated by predation and transduced as a wound response [3]. Local and systemic extracellular inducers of the signal pathway are released by injury. Current evidence suggests that the production of the inhibitors occurs via the octadecanoid pathway, which catalyzes the breakdown of linolenic acid and the formation of jasmonic acid to induce protease inhibitor gene expression [4–6]. Altogether, these findings indicate that plants defend themselves either directly by inducing the expression of compounds with insecticidal activity, such as the PIs. An effective plant defense against pests is most critically dependent on the efficacy of the effectors that mediate resistance (i.e. inhibitors produced by the plant that target proteases essential for the growth and development of the herbivore). Therefore there is need to identify and study the plant inducible insecticidal compounds such as PIs that have the potency to significantly retard the growth of herbivores.

Using naturally occurring plant PIs to target insect digestive enzymes has received serious consideration as a mean of insect pest management. Many studies on the interactions between insect digestive enzymes and plant PIs have been carried out with an objective of identifying potential inhibitors of insect proteinases [7,8]. Several identified plant PIs have shown to reduce the growth rates in larvae of Lepidopteran insect species [9,10]. Recently, Alves et al., [11] identified nine novel polypeptides that showed trypsin inhibitor activity from the crude extract of bean (*Phaseolus vulgaris*) seeds. A Bowman-Birk PI that strongly inhibited *Manduca sexta* trypsin and chymotrypsin like activity was purified and characterized from the seeds of black gram (*Vigna mungo*) [12]. Two Kunitz-type inhibitors with activity against trypsin and papain were isolated and characterized from the seeds of *Pithecellobium dumosum* (Jurema Branca) [13]. Both inhibitors showed their effectiveness against digestive enzymes of the larvae from diverse insect orders such as Lepidoptera, Coleoptera and Diptera [13]. Macedo et al., [10] studied the efficacy of *Adenanthera pavonina* trypsin inhibitor against the larvae of an *Anagasta kuehniella* (Zeller) (Lepidoptera: Pyralidae) and is found that *A. pavonina* trypsin inhibitor retard growth of *A. kuehniella* larvae [10]. Significant reduction in the growth and development was observed in the larvae of *Pieris rapae* and *Trichoplusia ni* when they reared on an artificial diet containing extracted cabbage foliage PI [14].

Several previous publications report the characterization of PIs from the seeds of pigeon pea [12,15–17]. Two PIs from pigeon pea seeds were purified and characterized according to their biochemical properties and inhibitory specificity [15]. PIs active

* Corresponding author. Tel.: +91 (0) 240 2403302x14; fax: +91 (0) 240 2403113/2403335.
E-mail address: vkhbiochem@gmail.com (V.K. Hivrale).

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against insect midgut trypsin-like proteinases were found in the seeds of 14 cultivars and eight wild types of pigeon pea [18]. A Bowman-Birk PI was characterized from the seeds of pigeon pea and the isolated inhibitor significantly inhibited midgut trypsin-like proteinases activity of *M. sexta* [12]. All these studies report the presence of PIs in the seeds of pigeon pea but no one has reported the presence of inducible PI in the leaves of pigeon pea. Inducible proteinase inhibitor proteins in plant leaves, when ingested by polyphagous insects, interact with proteases of the insect midgut and lead to an arrest of growth and development, and occasionally to death [3,19]. Since inducible plant PIs are important in conferring resistance to herbivorous insects, it is essential to search this type of PIs in pigeon pea. That will be beneficial in making the pigeon pea resistant to insect pests.

2. Material and methods

2.1. Chemicals

The following chemicals were obtained from Sigma–Aldrich, St. Louis, MO, (USA): Bovine trypsin, bovine chymotrypsin, N-α benzoyl-DL- arginine *p*-nitroanilide (BA*p*NA), methyl jasmonate (MeJA) and lanoline. Molecular weight markers were obtained from Genei, Bangalore, Karnataka State, India. Chemicals for electrophoresis were purchased from Merck, Germany and Sisco research laboratory (SRL), Mumbai, Maharashtra State, India. X-ray films were purchased from Selvas photographics, Silvassa, India.

2.2. Plant material and growth

Seeds of two pigeon pea varieties i.e. ICPW87 (susceptible) and ICPW332 (resistant) were obtained from International Crops Research Institute for Semi Arid Tropics (ICRISAT) Patancheru, Hyderabad, India. These varieties were chosen on the basis of their susceptibility and resistant to *Helicoverpa armigera*. The morphological and chemical characteristics associated with susceptibility and resistant in these verities are described by Sharma et al., [20]. It is described that, glandular trichomes on the calyxes and pods were associated with susceptibility to *H. armigera*, while the non-glandular trichomes were associated with resistance to this insect. Expression of resistance to *H. armigera* was also associated with low amounts of sugars and high amounts of tannins and polyphenols [20]. Therefore, we used one susceptible and one resistant variety of pigeon pea to check the difference in PI activity between these two varieties. Seeds were germinated for 48 h in a tray layered with wet filter papers and seedlings were transferred to earthen pots filled with a mixture of soil and farm yard manure (10:1). Plants were grown in a growth chamber with an 18 h (28 °C)/6 h (24 °C) light (300 mE)/dark cycle.

2.3. Plant treatments

The treatment of wounding and MeJA has been given to the plants to artificially induce the defense response. Wounding was carried out by dissecting needle and MeJA was applied to leaf surface by mixing it with lanoline. The purpose of using lanoline is that it can be used as adherent since MeJA can readily evaporate when directly applied to the leaf surface [21]. A 20 mL quantity of lanoline was poured into centrifuge tubes and 200 μL of MeJA (100 μM) was added into it and the mixture was rapidly stirred by glass rod. The mixture (5 mL to each petri-dish) was poured into petri-dishes, petri-dishes were sealed and mixture was allowed to solidify so it can be remained adhered to leaf surface. The mixture was applied to the stem or abaxial surface of the midrib of a leaf of plant by using a spatula [21]. Control plants were kept aside at another location (separate room) to avoid airborne contact with MeJA because MeJA which mixed with air can induce defense compounds when it makes contact with plant leaves. About 20 μL mixture was applied to each leaf. Three treatments of MeJA were carried out to pigeon pea plants at 50, 100, and 150 d from the date of sowing. The intervals of the treatment were selected according to the life cycle of the plant which is of about 180 d. Wounding was carried out by dissecting needle on the surface of the leaf. Intervals of wound treatments were similar to the MeJA application. Thirty plants were treated with wounding and other thirty plants were separately treated with MeJA. Wound treated plants were not treated with MeJA and vice versa. In the preliminary experiments the PI activity was measured at different time intervals such as 6, 12, 18, 24, 30, 36, 42, 48 h after application. Our preliminary studies indicated that the induction of PI was highest at 24 h after treatment therefore the leaves were harvested at the stage where the PI induction was highest (24 h). The leaves that directly treated with MeJA and wounding were considered as local leaves and their distal untreated leaves were considered as systemic leaves. Leaves harvested from the plants kept at another location were used as control. All the harvested leaves were immediately used for extraction.

2.4. Preparation of leaf extract

Separately harvested leaves were immediately weighed and homogenized with pre-chilled mortar and pestle in 1:6 (w/v) volumes of ice-cold 0.1 M Tris–HCl buffer pH 7.8. The homogenate was centrifuged (Sigma table top centrifuge 4-16K) at 10,000 *g* at 4 °C for 20 min. The supernatant was collected and divided into 2 mL aliquots (10 aliquots each) and stored at −20 °C until use. Aliquots were prepared to keep supernatant fresh and avoid contaminations. Protein concentration in supernatant was estimated by the method of Lowry using bovine serum albumin as standard [22].

2.5. Pigeon pea PI: induction and its characterization

2.5.1. Electrophoretic profile of PI from Different growth stages

PI from different growth stages of plants (50, 100 and 150 d) from the two cultivars of pigeon pea were separated by electrophoresis. Leaf extracts of untreated (20 μg of protein), MeJA treated (20 μg of protein) and wounded plants (20 μg of protein) were loaded on 10% native polyacrylamide gels and visualized by using gel X-ray film contact print technique [23] which is commonly used to detect plant protease inhibitors. After electrophoresis the gel was equilibrated with 0.1 M Tris–HCl buffer pH 7.8 and placed in 0.1 mg/mL trypsin or chymotrypsin solution (prepared in 0.1 M Tris–HCl buffer pH 7.8) for 15 min at 37 °C. The gel was then gently washed with fresh buffer and placed on an undeveloped X-ray film for 5–10 min. Hydrolysis of gelatin was visually monitored. At the end of incubation period gel was removed and X-ray film was washed gently with warm water to detect the trypsin/chymotrypsin inhibitor activity bands. The experiment was repeated three times with three replicates each at three different treatments.

2.5.2. Partial purification and molecular weight determination of PI

Partial purification of PI was achieved by preparative electrophoresis. Leaf extract (10 mL) from MeJA treated plants was loaded on 4% stacking gel. Electrophoresis was carried out at constant current of 20 mA for 4 h using Bio-Rad electrophoresis system. After electrophoresis a vertical strip of gel was cut and processed to detect PI activity band on X-ray film as described earlier. A horizontal strip of the remaining gel corresponds to PI activity band on

X-ray film was excised and kept overnight at −20 °C and the sample was eluted. The preparative electrophoresis was repeated 4–5 times to obtain sufficient amount of PI. Molecular weight of partially purified PI was determined with 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to the procedure of Laemmli [24]. PI sample (40 μg) obtained after preparative electrophoresis and molecular weight markers were mixed with SDS sample buffer containing dithiothreitol (DTT). The electrophoresis was carried out at constant current of 20 mA for 4 h. After electrophoresis the gel was stained with Coomassie Brilliant Blue (CBB) R-250 and protein bands were visualized.

2.6. Interaction of inducible PI with H. armigera gut proteinases (HGPs)

2.6.1. Extraction of HGPs

H. armigera larvae were obtained from International Crop Research Institute for Semi Arid Tropics (ICRISAT) Patancheru, Hyderabad, India. The fourth to fifth instar larvae (100) were dissected and midguts were isolated. The midgut tissues were homogenized with 1:6 (w/v) volumes of 0.1 M glycine-NaOH buffer pH 9.6. The homogenate was centrifuged at 10,000 *g* for 20 min at 4 °C and supernatant was used as a source of *H. armigera* gut proteinases (HGP). Protein in the extract was estimated by Lowry's method [22] with bovine serum albumin (BSA) as standard.

2.6.2. Inhibition of HGP isoforms

Inhibition analysis of HGP isoforms was carried out using gelatin reverse zymography [25]. Purified PI (5 μg) was pre-incubated with HGPs (20 μg of protein) for 30 min at 37 °C. The mixture and HGP without leaf extract (control) were loaded on 10% native PAGE and electrophoresis was carried out at constant current of 20 mA for 4 h. HGPs without leaf extract were served as standard. After electrophoresis the gel was washed with distilled water and equilibrated in 0.1 M Glycine-NaOH buffer pH 9.6. After equilibration the gel was placed in 1% (w/v) gelatin (prepared in the same buffer) and incubated for 2 h at 37 °C. Then the gel was stained with a protein binding dye CBBR-250 which has capacity to stain proteins with high band visibility. Further the gel was destained to visualize proteinase activity isoforms. The experiment was repeated three times with three replicates each.

2.6.3. Inhibition of total HGP and trypsin-like activity

Total HGP activity inhibition assay was carried out using casein as substrate [26]. Purified PI (5 μg) was pre-incubated with HGP (20 μg of protein) for 30 min at 37 °C. The respective reactions were carried out for 20 min at 37 °C in 0.1 M Glycine-NaOH buffer pH 9.6 with 2 mL of 0.5% casein (prepared in same buffer) and 40 μL of pre-incubated trypsin–inhibitor reaction mixture. After 20 min, the reactions were terminated by adding 3 mL of 5% trichloro acetic acid (TCA). TCA soluble peptides were measured at 280 nm.

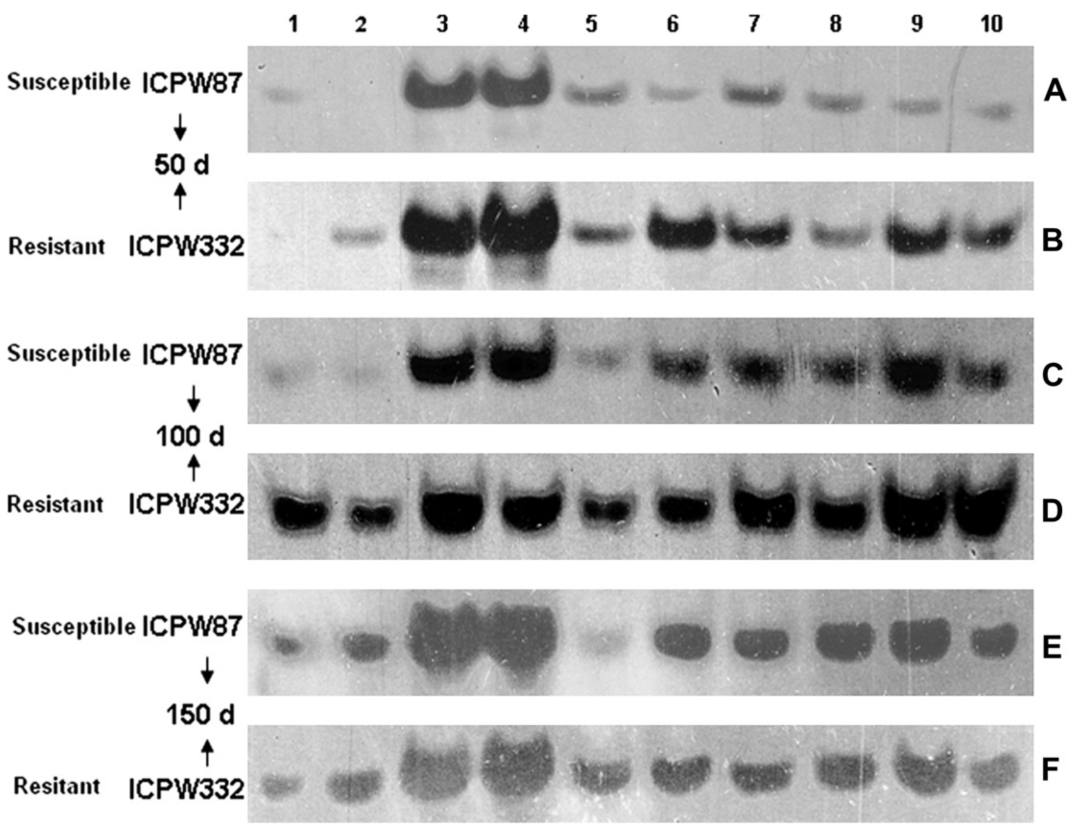

Fig. 1. Detection of induced PI at different growth stages of pigeon pea plants. Local and systemic leaf extracts of control, MeJA treated and wounded plants were electrophoretically processed to detect PI on X-ray film. Two biological replicates of every leaf extract were resolved on 10% native PAGE and PI was electrophoretically separated. Lane 1 control untreated plant replicate 1 (20 μg), lane 2 control untreated plant replicate 2 (20 μg), lane 3 local leaf extract of MeJA treated plant replicate 1 (20 μg), lane 4 local leaf extract of MeJA treated plant replicate 2 (20 μg), lane 5 systemic leaf extract of MeJA treated plant replicate 1 (20 μg), lane 6 systemic leaf extract of MeJA treated plant replicate 2 (20 μg), lane 7 local leaf extract of wounded plant replicate 1 (20 μg), lane 8 local leaf extract of wounded plant replicate 2 (20 μg), lane 9 systemic leaf extract wounded plant replicate 1 (20 μg), lane 10 systemic leaf extract wounded plant replicate 2 (20 μg). A – PI activity pattern in the pigeon pea variety ICPW87 upon treatments at 50 d after sowing. B – PI activity pattern in the pigeon pea variety ICPW332 upon treatments at 50 d after sowing. C – PI activity pattern in the pigeon pea variety ICPW87 upon treatments at 100 d after sowing. D – PI activity pattern in the pigeon pea variety ICPW332 upon treatments at 100 d after sowing. E – PI activity pattern in the pigeon pea variety ICPW87 upon treatments at 150 d after sowing. F – PI activity pattern in the pigeon pea variety ICPW332 upon treatments at 150 d after sowing.

H. armigera gut trypsin-like activity inhibition assay was carried out using synthetic chromogenic substrate BA*p*NA which is a specific substrate for trypsin-like protease and librates yellow colored compound *p*-nitroaniline after the action of trypsin [27]. Purified PI (5 μg) was pre-incubated with *H. armigera* gut extract (20 μg of protein) for 30 min at 37 °C. The respective reactions were carried out for 20 min at 37 °C in 0.1 M Glycine-NaOH buffer (pH 9.6) with 200 μL of 10 mM BA*p*NA and 40 μL of reaction mixture (PI + HGP). After 20 min the reactions were terminated by adding 150 μL of 30% acetic acid. The rate of production of *p*-nitroaniline was measured at 410 nm. Assay was carried out at least three times. Proteinase activity was defined as production of one μM of *p*-nitroaniline/mg of protein/min at 37 °C. The experiment was repeated three times with three replicates each.

2.7. Statistical analyses

All the experiments were carried out at least three times with three biological or technical replicates; *t* tests were performed to verify the significance of the observed differences in HGP activities in control and after incubation with PI. The statistical analysis was performed using SPSS 15.0 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Local and systemic induction of PI isoforms at different growth stages of plants

PI activity observed to be induced in local as well as systemic leaves after first treatment (at 50 d from sowing) of wound and MeJA (Fig. 1A and B). PI activity was rather strong in MeJA treated local leaves as compared to control and wound treated leaves (Fig. 1A, B lane 3 and 4). Similarly the PI activity was higher in wound treated local as well as systemic leaves as compared to control leaves (Fig. 1A, B lane 7, 8, 9 and 10) After second treatment (at 100 d from sowing) similar induction pattern of PI activity was observed (Fig. 1C and D). After third treatment (at 150 d from sowing) the induction pattern of PI was similar in susceptible as well as resistant variety (Fig. 1E and F). We observed somewhat high PI activity in the control samples of 100 and 150 d old plants as compared to 50 d old plants (Fig. 1D–F lane 1 and 2).

3.2. Detection of induced trypsin and chymotrypsin Inhibitor

Along with detection of PIs at different growth stages we separately detected the trypsin inhibitor (TI) and chymotrypsin inhibitor (CTI) activities. The main purpose was to check whether the induced PI can inhibit trypsin as well as chymotrypsin. We observed that the detected PI isoform showed dual inhibitory activity i.e. TI as well as CTI (Fig. 2A and B). Highest CTI activity was also found in leaf extracts of MeJA treated plants, whereas wounding was also responsible for significant induction of CTI (Fig. 2B).

3.3. Partial purification and molecular weight determination

The purification of induced PI was achieved by preparative electrophoresis. The molecular weight of pigeon pea purified PI was estimated to be ∼17.7 kDa (Fig. 3).

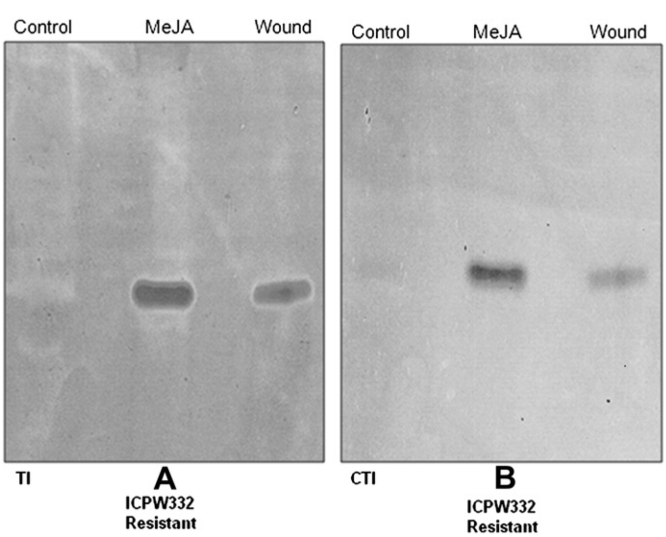

Fig. 2. Induction profile of trypsin inhibitor (TI) and chymotrypsin inhibitor (CTI). A and B – TI and CTI respectively. Leaves of pigeon pea variety ICPW332 were treated with MeJA and wounding after 100 d from the date of sowing and local leaf extract was used for the experiment. A and B – Leaf extracts of untreated, MeJA treated and wounded leaves were resolved on 10% native PAGE and visualized on X-ray film. Highest induction of TI and CTI isoforms was observed in MeJA treated plants. Lane 1 control untreated plant's leaf extract (15 μg), lane 2 MeJA treated plant's leaf extract (15 μg), lane 3 leaf extract of wounded plants (15 μg).

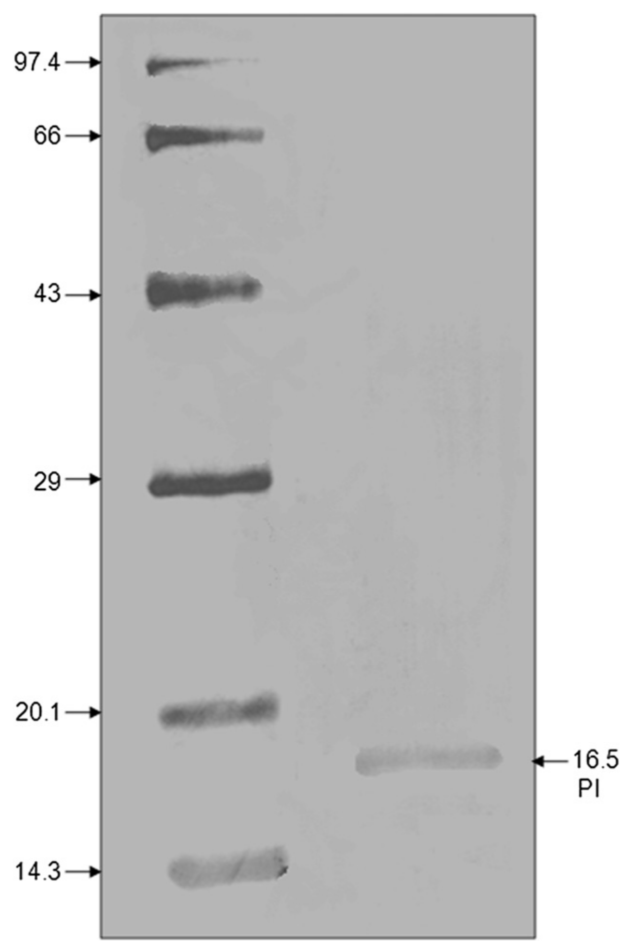

Fig. 3. Molecular weight determination of pigeon pea defensive PI. Purified PI (20 μg) with molecular weight markers was resolved on 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Prior to electrophoresis PI sample and molecular weight marker were mixed with sodium dodecyl sulfate (SDS) sample buffer containing dithiothreitol (DTT). After electrophoresis the gel was stained with Coomassie Brilliant Blue (CBB) R-250 and protein bands were visualized. Lane 1 molecular weight markers proteins and their molecular weight in kDa, lane 2 purified PI.

3.4. Inhibition of HGPs

Inhibition potency of pigeon pea inducible defensive PI was checked against HGP. Among the ten HGP isoforms detected HGP 1, 2, 8 and 10 were completely inhibited by purified PI whereas HGP 3, 5, 6 and 7 were partially inhibited by purified PI (Fig. 4A). Inhibition of total HGP activity and gut trypsin-like activity was estimated by assays using casein or BA*p*NA as substrate. Total HGP activity was strongly inhibited by purified PI. Significant induction of total HGP activity was recorded with purified PI as compared to control ($p = 0.03678$) (Fig. 4B). *H. armigera* gut trypsin-like activity was also strongly inhibited by purified PI as compared to control ($p = 0.00816$) (Fig. 4C).

4. Discussion

Most storage organs of plants such as seeds and tubers contain 1–10% of their total proteins as PIs. Plant PI have been extensively studied, and strategies for developing resistance against insect pests using PI have been suggested [1,28–31]. A study of the induction and accumulation of PI in pigeon pea leaves, which are extensively damaged by *H. armigera* feeding, was needed. Furthermore, it was necessary to study PI induction during plant development and the interaction of these inhibitors with insect gut proteinases. Such studies will help to devise strategies for obtaining sustainable practical solutions to insect-pest infestation in legumes.

Although considerable information is available about wound-inducible PI of tobacco and tomato [32–34], little is known about the wound inducibility of PI in leaves of pigeon pea plants. Since the inhibitors are synthesized as part of the plant's defense response, information about the induction and levels of PI in leaves in response to wounding at different stages of development would be valuable. Our results constitute the first report, to our knowledge, on the induction of pigeon pea PI during the different stages of plant growth. In the present investigation, we have detected and characterized wound and MeJA inducible PI from leaves of pigeon pea. Single band of PI activity was detected in the leaves of pigeon pea at different growth stages. The activity of PI is considerably induced after the treatment of wounding and MeJA. We observed that induction of PI was higher in MeJA treated leaves as compared to wound treated leaves. We observed somewhat high PI activity in the control samples of 100 and 150 d old plants as compared to 50 d old plants. This might be a plant's normal physiology to have higher PI activity as the plant become older. Again another possibility is that at this stage (100–150 d) plant might have severe insect attack therefore plant need to have high PI activity. From the induction pattern of PI it seems that the pigeon pea has precise defense mechanism against herbivores. Our results are consistent with various previous studies. Several studies have demonstrated that PI proteins are specifically produced in the plant upon biotic stress and protect the plant tissue from the damage [1,32,33,35]. PIs are the most exploited class of plant defense proteins for their use in developing insect resistance in plants [1,2].

The inducible PI from pigeon pea leaves has been partially purified and characterized on the basis of its biochemical properties. Molecular weight of induced PI was found to ~16.5 kDa. Based on the molecular weight estimates of ~16.5 kDa and its bi-specific inhibitory activity towards trypsin and chymotrypsin, pigeon pea

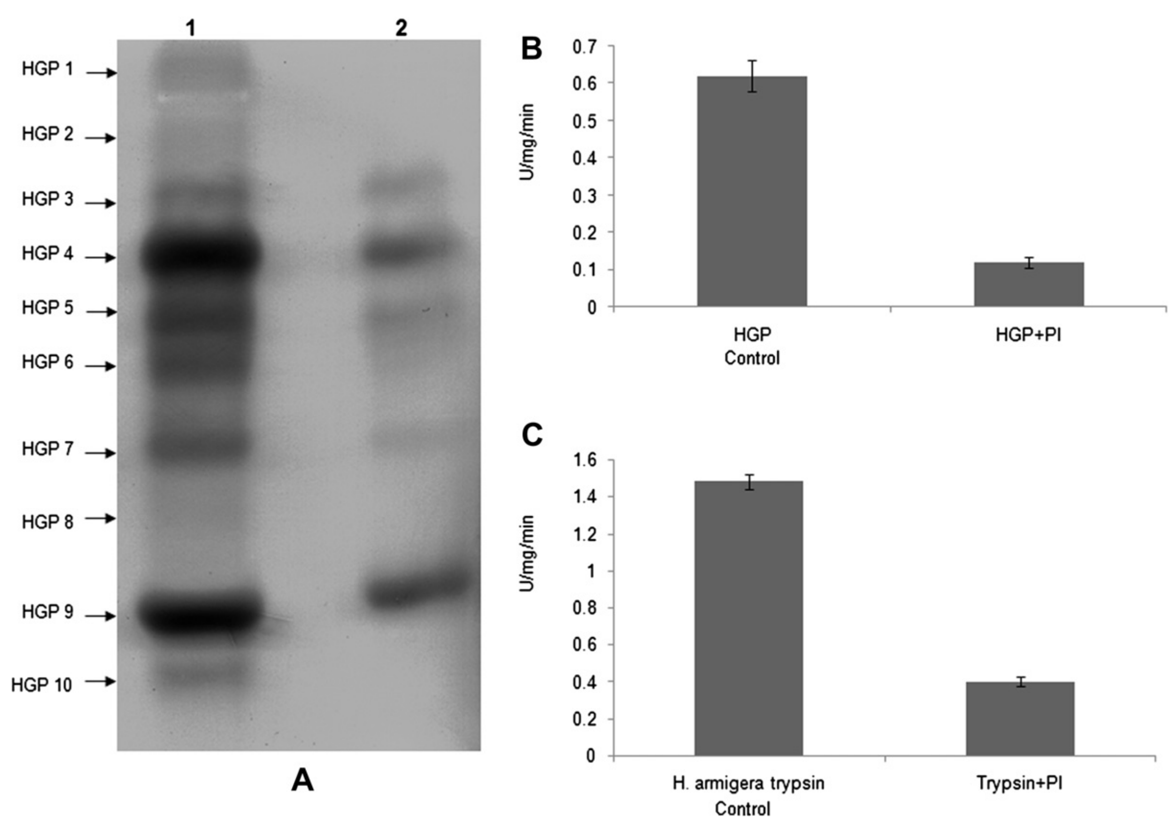

Fig. 4. In vitro interaction of pigeon pea inducible defensive PI with HGPs. A – Inhibition of HGP isoforms by pigeon pea inducible defensive PI. HGP were pre-incubated with purified PI (5 µg) and the mixture was loaded on 10% native page and visualized on X-ray film. Lane 1 HGPs, lane 2 HGPs + Purified PI. B – Inhibition of total HGPs activity by pigeon pea PI. Total HGPs activity inhibition assay was carried out using casein as substrate. Purified PI (5 µg) was pre-incubated with *H. armigera* gut extract and assay was carried out for 20 min at 37 °C. Vertical bars represent standard error. Significant differences were calculated by Student's *t* test. C – Inhibition of *H. armigera* trypsin-like activity by pigeon pea PI. Trypsin-like activity inhibition assay was carried out using BA*p*NA as substrate. Purified PI (5 µg) was pre-incubated with *H. armigera* gut extract and assay was carried out for 20 min at 37 °C. Vertical bars represent standard error. Significant differences were calculated by Student's *t* tests.

defensive PI may belongs to Bowman-Birk protease inhibitor family [1,18,28]. The inhibitors belonging to this family were typically observed to be inhibitors of serine proteinases such as trypsin and chymotrypsin [1]. Previously characterized inhibitors from the seeds of pigeon pea found to have lower molecular weights and bispecific inhibitory activity towards trypsin and chymotrypsin [15,18].

Interesting finding of the present investigation is the presence of single inducible PI isoform in pigeon pea leaves. However several studies demonstrated the presence of quite a few inhibitor isoforms in the seeds of pigeon pea species [7]. Pigeon pea seeds contain proteinaceous inhibitors of trypsin, chymotrypsin and amylases [36,37], which constitute one of the parts of defense machinery. Biochemical characterization of pigeon pea PIs has revealed that these are small molecules having inhibitory activity against trypsin and chymotrypsin [15]. Previously, seven isoforms of trypsin/chymotrypsin inhibitors and two isoforms of trypsin inhibitors have been reported from pigeon pea seeds [23]. Similarly more than a few inhibitors were found in the several other plant species [1,38]. Seven isoforms of PI were isolated from leaves of MeJA treated pepper plants [39]. Pearce et al., [40] isolated six small molecular weight, wound-inducible trypsin and chymotrypsin inhibitor proteins from tobacco (*Nicotiana tabacum*) leaves. Dry mature seeds of winged bean (*Psophocarpus tetragonolobus*) contain several proteinase inhibitors [41]. More than one PI isoforms have been observed in the seeds of pigeon pea [7]. However, in the present study a single PI isoform was detected in the leaves of pigeon pea. One possible explanation is that usually insect moths lay eggs on the lower side of the leaves and when the neonate larvae arises they start to feed on leaves and gradually move towards the higher parts of the plant as they grow [42]. Thus the presence of single inhibitor in the pigeon pea leaves may sufficient to act on neonate larvae. As the larvae grow they prefer to feed on pods and seeds. Thus the plants may produce more PI isoforms with varying specificity to counteract with diverse midgut proteinases of the insect larvae. It is important to note that the presence of one or more PIs in a particular plant species is also depends on their role in the regulation of internal proteinase activity since PI are not used only as a defense tool by plants [43].

Our aim was to establish to what extent and how pigeon pea inducible defensive PI could affect digestive proteases of insects. *H. armigera* was selected as the model insect, because it primarily uses serine proteinases for proteolytic digestion [41] and *H. armigera* is also a serious pest of pigeon pea [7,44]. Furthermore, *H. armigera* is a polyphagous pest attacking a variety of commercially important crops including chickpea, cotton, tomato and several other plants [45]. The obtained results demonstrate that pigeon pea inducible PI is effective for inhibition of both total HGP activity and trypsin like activity from *H. armigera* midgut extract. Our results also indicate that the inducible PI able to inhibit trypsin as well as chymotrypsin. PIs having the inhibitory activity against trypsin and chymotrypsin are important because most of the lepidopteran insect gut, serine proteinase (trypsin and chymotrypsin) activities are dominant [44]. Similarly in *H. armigera*, most of the proteinase isoforms found in the gut are trypsin or chymotrypsin-like. Therefore the isolated bi-specific PI of pigeon pea can inhibit both of the enzyme activity (trypsin and chymotrypsin) of *H. armigera* gut proteinases.

While describing the ideal PI for developing insect resistant plants several researchers have proposed that the identified PIs should have strong inhibitory potential and stability against insect gut proteinases [28,29,31,38,46]. Our study indicates that pigeon pea PI effectively inhibit trypsin, chymotrypsin, total HGP and *H. armigera* gut trypsin like activity. Pigeon pea defensive PI effectiveness would rely on its ability to inhibit two different types of proteolytic activity (trypsin and chymotrypsin). Clearly, bispecific plant PIs would offer advantages since they can able to control different proteinase of a given insect pest.

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